

# Cross-linking of *SsoII* restriction endonuclease to cognate and non-cognate DNAs

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**Abstract** Specific and non-specific interactions of *SsoII* restriction endonuclease (R-*SsoII*) were probed by the method of covalent attachment to modified DNA containing an active monosubstituted pyrophosphate internucleotide bond instead of a phosphodiester one. R-*SsoII* with six N-terminal His residues was shown to be cross-linked to duplexes with this type of modification, either containing or not the recognition sequence. Competition experiments with covalent attachment of R-*SsoII* to activated DNAs demonstrated the similar affinity of the enzyme to cognate and non-cognate DNAs in the absence of cofactor, Mg<sup>2+</sup> ions.

**Key words:** DNA-protein interaction; Restriction endonuclease; Duplex with active internucleotide group; Covalent attachment

## 1. Introduction

Recently it was established that a number of restriction endonucleases require Mg<sup>2+</sup> ions, not only for catalysis but also for the specificity of substrate recognition [1]. For example, restriction endonuclease *EcoRV* interacts with cognate and non-cognate DNAs with nearly equal affinity in the absence of cofactor [2], as revealed by nitrocellulose binding or electrophoretic mobility shift assay. In the present paper we investigated the specific and non-specific interactions of the restriction endonuclease *SsoII* (R-*SsoII*) with DNAs by the method of covalent attachment of the enzyme to activated DNA. We chose DNA duplexes containing a monosubstituted pyrophosphate internucleotide bond instead of a phosphodiester one in the enzyme recognition site as reagents for affinity modification of R-*SsoII*. These activated DNA duplexes were recently shown to successfully modify the active centers of restriction endonucleases *RsrI*, *EcoRI* [3], *EcoRII* [4,5] and *MvaI* [5]. Covalent attachment may occur on the binding or catalytic step depending on cross-linking conditions.

## 2. Materials and methods

### 2.1. Enzymes and oligonucleotides

R-*SsoII* (10 000 U/ml) was purified from *E. coli* M15 cells carrying the isopropyl-β-D-thiogalactopyranoside (IPTG)-inducible overexpression plasmid pQE.Sso9 [6]. This plasmid contained the R-*SsoII* gene [7] in the pQE9 tac expression vector (DIAGEN GmbH, Germany). The

*SsoII* endonuclease encoded by pQE.Sso9 plasmid possessed an N-terminal 6×His affinity tail, making it possible to purify the enzyme to virtual homogeneity by one-step Ni-chelate affinity column chromatography. The introduced amino acid substitutions do not change R-*SsoII* recognition and cleavage properties if compared with the unmodified enzyme obtained earlier [8].

Oligonucleotide precursors were synthesized by T.S. Oretskaya, E.M. Volkov and E.A. Romanova as described in [9]. DNA duplexes with a monosubstituted pyrophosphate internucleotide bond for cross-linking to R-*SsoII* were synthesized as described in [10]. DNA duplex I with an ethoxy or isopropoxy group and DNA duplexes II and III with an ethoxy group in modified internucleotide linkage were obtained (Table 1). DNA duplexes were <sup>32</sup>P-labeled by T4 polynucleotide kinase. <sup>32</sup>P label was at the monosubstituted phosphate group of the modified internucleotide linkage of the DNA duplex modified strand (see formulae) and at the 5' end of the non-modified strand. Cleavage analysis of DNA duplexes by R-*SsoII* was performed as described in [11].

### 2.2. Cross-linking experiment

Cross-linking of R-*SsoII* (concentration per monomer 1.4×10<sup>-6</sup> M) to substrates I–III (concentration per duplex 1.8×10<sup>-7</sup> M) was performed in 20 μl of buffer A: 10 mM *N*-methylimidazole (MeIm), pH 7.5, 50 mM NaCl, 0.1 mM dithiothreitol (DTT) and 0, 0.15 or 15 mM MgCl<sub>2</sub>, or B: 10 mM Tris-HCl, pH 7.5, 50 mM NaCl, 0.1 mM DTT and 0, 0.15 or 15 mM MgCl<sub>2</sub> at 37°C for 18 h. DNA duplexes I–III contained <sup>32</sup>P-label only at the monosubstituted phosphate group of modified internucleotide linkage (see formulae). Reactions were followed by 0.1% SDS–10% PAGE [12] after heating samples in 0.1% SDS–2-mercaptoethanol solution at 95°C. The gels were analyzed by autoradiography and staining with Coomassie blue. The coincidence of the radioactive band and the band containing protein proved the formation of the DNA-enzyme covalent complex. Cross-linking yield was determined as the ratio of the radioactivity of the DNA-enzyme covalent complex to the total radioactivity of the conjugate and the unbound DNA. The average results for cross-linking of R-*SsoII* to duplexes I and II are reported in Table 2.

### 2.3. Competition inhibition of R-*SsoII* cross-linking to duplexes with a monosubstituted pyrophosphate internucleotide bond

Competition inhibition of R-*SsoII* cross-linking to duplexes I and II was studied in the presence of increasing amounts of unlabeled DNA duplexes IV and V added to reach ratios of molar concentrations of inhibitor and reagent equal to 0.5, 1, 2, 3, 4, 5, 7, 10, 14, 21, 28. Conditions of the reactions: buffer B, 37°C, 18 h.

## 3. Results and discussion

R-*SsoII* recognizes the double stranded sequence



degenerated at the central position and cleaves it as shown by arrows.

For the affinity modification of R-*SsoII*, we used <sup>32</sup>P-labeled DNA duplexes I and II containing the recognition site of this

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Abbreviations: MeIm, *N*-methylimidazole; DTT, dithiothreitol

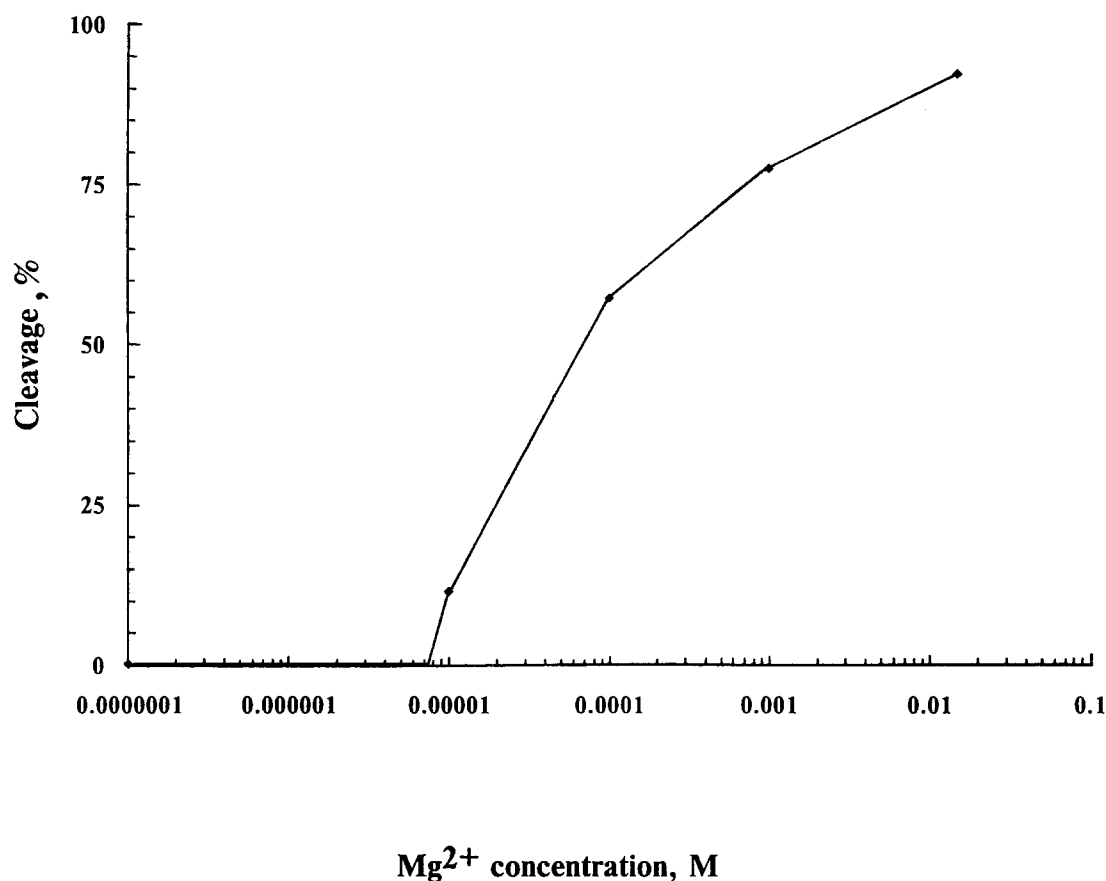
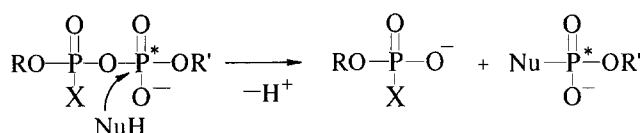


Fig. 1. Dependence of 14-mer canonical DNA duplex cleavage extent by R*Sso*II on  $Mg^{2+}$  ion concentration. Cofactor concentration is added in logarithmic scale. 37°C, 1 h, buffer B.

enzyme (Table 1),  $^{32}\text{P}$  label being located in the disubstituted phosphate group of the pyrophosphate. DNA duplex I contained the activated pyrophosphate group in the middle of the R-*SsoII* recognition sequence (marked in bold). In duplex II, the monosubstituted pyrophosphate bond was adjacent to the recognition site and coincided with the R-*SsoII* scissile bond. Covalent attachment of the nucleophilic agent to the modified strand of the duplex follows the scheme



where NuH is a nucleophilic agent, RO and R'O are oligonucleotide residues, X is a non-nucleotide substituent, and P\* is the  $^{32}\text{P}$  label.

Ethoxy or isopropoxy groups were chosen as non-nucleotide substituents. The introduction of an additional methyl group into the non-nucleotide substituent of the modified internucleotide linkage does not influence the interaction between activated DNA and proteins (unpublished results). The nucleophilic amino acid side chains of R-*SsoII* are supposed to be attached to oligonucleotide \*pTGGTGGT from duplex I or \*pCCTGGATCCG from duplex II during the cross-linking reaction [10].

The capability of R-*SsoII* to bind DNAs with modification of the sugar-phosphate backbone was demonstrated by the gel retardation assay (data not shown). Introduction of a mono-

substituted pyrophosphate bond does not prevent the hydrolysis of duplexes I and II by R·SsoII (Table 1).

The data on cross-linking of oligonucleotides from duplexes I and II to R-*Sso*II are adduced in Table 2. One can see that the yield of cross-linking varies from 2 to 22% in buffer B (Tris-HCl buffer) in the absence of cofactor,  $Mg^{2+}$  ions. We tried to get higher yields of cross-linking with the help of the nucleophilic catalyst MeIm which was previously shown to increase the efficiency of restriction endonuclease attachment to the same type of activated DNA [3-5]. But the presence of MeIm did not result in an increase of the extent of cross-linking of R-*Sso*II to duplexes I and II in comparison with

Table 1  
R·SsoII hydrolysis of DNA duplexes I and II in buffer B

N	DNA duplex	Strand	Cleavage, %
I*)	$  \begin{array}{c}  \text{AlkO} \quad \quad \quad \text{O}^- \\    \quad \quad \quad   \\  5' \text{ ACCTACC-O-P-O-P-O-TGGTGGT } 3' \\  \quad \quad \quad    \quad \quad    \\  \quad \quad \quad \text{O} \quad \quad \text{O} \\  \text{.....} \quad \quad \quad \text{.....} \\  3' \text{ TGGATGG} \text{-----} \text{ACCACCA } 5'  \end{array}  $	a  b	29  34
II	$  \begin{array}{c}  \text{C}_2\text{H}_5\text{O} \quad \quad \quad \text{O}^- \\    \quad \quad \quad   \\  5' \text{ GTCACT-O-P-O-P-O-CCTGGATCCG } 3' \\  \quad \quad \quad    \quad \quad    \\  \quad \quad \quad \text{O} \quad \quad \text{O} \\  \text{.....} \quad \quad \quad \text{.....} \\  3' \text{ CAGTGA} \text{-----} \text{GGACCTAGGCA } 5'  \end{array}  $	a  b	**) 51

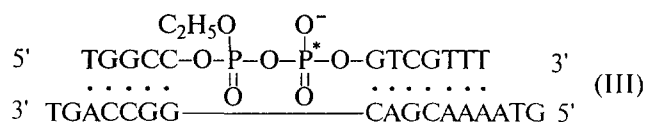
\*Alk= $i\text{C}_3\text{H}_7$ .

\*\*The extent of hydrolysis was not determined due to the coincidence of the scissile bond and the modified internucleotide bond.

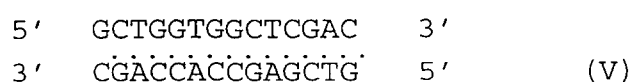
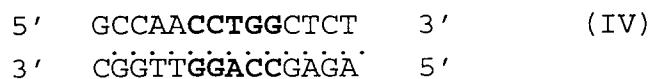
that in Tris-HCl buffer (Table 2).

There is a tendency that introduction of  $Mg^{2+}$  ions causes a decrease of cross-linking of R-SsoII to duplex I. Comparing data of Table 2 and Fig. 1, one can conclude that this decrease is connected with substrate hydrolysis at  $Mg^{2+}$  concentrations higher than  $1.5 \times 10^{-4}$  M. In the case where the modified internucleotide bond coincides with the scissile bond (duplex II), addition of  $Mg^{2+}$  ions does not influence the cross-linking yield.

To investigate the specificity of R-*Sso*II cross-linking to duplexes I and II we (i) checked the possibility of affinity modification of R-*Sso*II by activated DNA duplex III without the R-*Sso*II recognition site



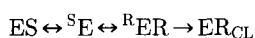
and (ii) tested the possibility of inhibition of cross-linking of reagents (DNA duplexes I and II) by non-modified duplexes with (IV) or without (V) recognition site



## Appendix

### Calculation of the ratio of constants of specific and non-specific association

Consider the case when two equilibria occur



where E is enzyme; S is substrate; R is reagent for cross-linking;  $ER_{CL}$  is product of covalent attachment of the enzyme to reagent; ER and ES are enzyme-reagent and enzyme-substrate complexes respectively.

Thermodynamic constants can be written as

$$K^R = \frac{[ER]}{[E][R]}; \quad K^S = \frac{[ES]}{[E][S]}$$

for enzyme-reagent and enzyme-substrate complexes respectively.

Their ratio will be equal to

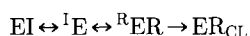
$$\frac{K^R}{K^S} = \frac{[ER][E][S]}{[ES][E][R]} = \frac{[ER][S]}{[ES][R]}$$

A 2-fold decrease of cross-linking indicates that half of the busy active centers are occupied by the substrate rather than reagent, in this case  $[ES]=[ER]$  and

$$\frac{K^R}{K^S} = \frac{[S]_{50}}{[R]_{50}}$$

where  $[S]_{50}/[R]_{50}$  is ratio substrate/reagent (i.e. duplex IV/duplex I) in the case where cross-linking efficiency is equal to 50% of the initial (or decreases 2-fold).

In the similar case



where I is non-cognate DNA inhibitor and EI is complex of the enzyme with non-cognate DNA inhibitor (i.e. duplex V),

$$\frac{K^R}{K^I} = \frac{[I]_{50}}{[R]_{50}}$$

where  $[I]_{50}/[R]_{50}$  is ratio inhibitor/reagent (i.e. duplex V/duplex I) in the case where cross-linking efficiency is equal to 50% of the initial (or decreases 2-fold). Distributing  $K^R/K^I$  by  $K^R/K^S$  we get

$$\frac{K^R/K^I}{K^R/K^S} = \frac{K^S}{K^I} = \frac{[I]_{50}/[R]_{50}}{[S]_{50}/[R]_{50}}$$

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